# Expression of Myostatin Pro Domain Results in Muscular Transgenic Mice

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ABSTRACT Myostatin, a member of the TGFβ family, negatively regulates skeletal muscle development. Depression of myostatin activity leads to increased muscle growth and carcass lean yield. In an attempt to down-regulate myostatin, transgenic mice were produced with a ribozyme-based construct or a myostatin pro domain construct. Though the expression of the ribozyme was detected, muscle development was not altered by the ribozyme transgene. However, a dramatic muscling phenotype was observed in transgenic mice carrying the myostatin pro domain gene. Expression of the pro domain transgene at 5% of β-actin mRNA levels resulted in a 17-30% increase in body weight (P < 0.001). The carcass weight of the transgenic mice showed a 22-44% increase compared with nontransgenic littermates at 9 weeks of age  $(16.05 \pm 0.67 \text{ vs. } 11.16 \pm 0.28 \text{ g in})$ males;  $9.99 \pm 0.38$  vs.  $8.19 \pm 0.19$  g in females, P < 0.001). Extreme muscling was present throughout the whole carcass of transgenic mice as hind and fore limbs and trunk weights, all increased significantly (P < 0.001). Epididymal fat pad weight, an indicator of body fat, was significantly decreased in pro domain transgenic mice (P < 0.001). Analysis of muscle morphology indicated that cross-sectional areas of fast-glycolytic fibers (gastrocnemius) and fast-oxidative glycolytic fibers (tibialis) were larger in pro domain transgenic mice than in their controls (P < 0.01), whereas fiber number (gastrocnemius) was not different (P > 0.05). Thus, the muscular phenotype is attributable to myofiber hypertrophy rather than hyperplasia. The results of this study suggest that the overexpression of myostatin pro domain may provide an alternative to myostatin knockouts as a means of increasing muscle mass in other mammals. Mol. Reprod. Dev. 60: 351-361, 2001. © 2001 Wiley-Liss, Inc.

**Key Words:** muscle mass; muscle fibers; TGF- $\beta$ ; ribozyme

### INTRODUCTION

Myostatin is a genetic determinant of skeletal muscle mass in the bovine and mouse. An 11-nucleotide deletion and a mis-sense mutation in the exon 3 of myostatin are responsible for the double muscling in

Belgian Blue and Piedmontese cattle, respectively (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Myostatin-null mice, generated by gene targeting, produce twice the muscle mass as wild-type mice (McPherron et al., 1997). Homozygous mutant mice were about 30% larger in body weight, and their muscles had larger cross-sectional fiber areas (hypertrophy) as well as more fibers number (hyperplasia) than their wild-type littermates. Those results suggest that the depressing myostatin activity by genetic manipulation could lead to enhanced muscle growth and meat production in livestock animals.

Myostatin, also known as growth and differentiation factor 8 (GDF-8), belongs to the transforming growth factor β (TGF-β) superfamily. Mature TGF-β proteins are generated by enzyme cleavage of the C-terminus of precursor molecules. The biologically active forms of TGF-β are dimers, and remain associated with their Nterminal propeptides through noncovalent interactions in the forms of latent TGF-β complexes (Gleizes et al., 1997). The biological activation mechanism from latent TGF-β complexes to mature growth factors is not well understood. However, a mutation or truncation of the cleavage site has been demonstrated to be an effective way to block activities of  $TGF-\beta$  family members (Gentry and Nash, 1990; Hawley et al., 1995). In transgenic mice, expression of a myostatin with a mutated cleavage site resulted in a decreased level of processed myostatin, and an increased level of uncleaved complex (Zhu et al., 2000). In COS-1 cell transient expression studies, the pro domain of TGFβ1 cDNA was able to form latent complexes with mature TGF-β1, inhibiting its biological activity (Gentry and Nash, 1990). Based on these observations, we reasoned that over expression of the pro domain of myostatin would interfere with myostatin function to negatively regulate skeletal muscle development, thus promoting muscle growth.

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Myostatin is highly conserved among species. The predicted myostatin proteins in human, rat, mouse, pig, chicken, and turkey, are identical in the biologically active carboxy-terminal region (McPherron and Lee, 1997; Gonzalez—Cadavid et al., 1998). Since myostatin has a similar function in the mouse and the bovine, it is likely that myostatin would also serve as a negative regulator of skeletal muscle in other livestock animals.

Genetic manipulation of myostatin in livestock to increase muscle mass is appealing. However, that is true only as long as other economically important traits are not compromised. Calving difficulty is widely observed in double-muscled cattle. Belgian Blue calves are routinely delivered by cesarean section because their enlarged muscles restrict movement through the relatively narrow pelvic girdle of the dam (Arthur, 1992). The calving difficulties have diminished producers' interest in using double-muscled cattle in their production herds. However, myostatin-null and transgenic mice with depressed myostatin activity had no apparent difficulties delivering their pups (McPherron et al., 1997; Zhu et al., 2000). This may be due to the litter-bearing nature of mice. Swine, like mice, are litter-bearing animals, and therefore parturition problems seen in cattle may not be a concern if myostatin activity were depressed in swine. Furthermore, a transgene might be designed to postpone onset of enhancement of muscle development thus avoiding parturition difficulties in cattle.

Down-regulation of gene function by knock-out approaches is not yet established in swine. In order to down-regulate myostatin activity in pigs, transgene constructs might be designed to reduce myostatin RNA, block myostatin protein production, or interfere with myostatin function. Ribozyme-based transgenes are an effective way of reducing target RNA in tissue culture (Haseloff and Gerlach, 1988), but have had a checkered record in transgenic animals. They have been successfully used in reduction of α-lactalbumin, glucokinase and \beta2-microglobulin mRNA in transgenic mice (Efrat et al., 1994; Larsson et al., 1994; L'Huillier et al., 1996). As a pilot study for a transgenic pig project, we have developed both ribozyme and myostatin pro domain approaches in an attempt to down-regulate myostatin activity. Expressing lines for both types of constructs were produced, but we detected no alteration in muscle growth in the ribozyme-carrying mice. However, the over-expression of myostatin pro domain DNA dramatically enhanced skeletal muscle development.

# MATERIALS AND METHODS Production of Transgenic Mice

**Ribozyme transgene.** The secondary structure of mouse myostatin mRNA was predicted using software MFOLD version 3.1 (Zuker et al., 1999). Four potential target sites for hammerhead ribozymes (Perriman et al., 1992) were identified within regions of the mRNA sequence that did not appear to be constrained

by secondary structure annealing. Hammerhead ribozymes were designed by inserting the consensus hammerhead motif (Haseloff and Gerlach, 1988) between flanking sequences complementary to each of the target sites in the mouse myostatin mRNA. Double stranded DNA fragments encoding the four individual ribozymes were created by annealing complementary synthetic oligonucleotides, and the individual ribozyme coding regions were ligated to one another to produce a multi-target ribozyme gene as described by Chen et al. (1992) and Leopold et al. (1996). The multi-target ribozyme gene was flanked by inverted repeats so as to position the ribozymes sequences in the center of a stable stem-loop structure (Lieber and Strass, 1995).

**Pro domain transgene.** The coding sequence for myostatin pro domain was obtained by PCR amplification of the 5'-flanking 0.8 kb fragment of mouse myostatin cDNA (Amino acid residue 1–266, Genbank accession number U84005). The T7 forward primer and reverse primer (5'-GCGGATCCTGAGCACCCACAGCG-3'), incorporating a Bam H I restriction site were used to generate GDF-8 cDNA for in-frame fusion with the FLAG epitope. The resulting cDNA was ligated into the pCR 2.1 vector (Invitrogene Carlsbad CA) and sequenced for verification.

Transgene construction. Both ribozyme and myostatin pro domain DNA were inserted into the pMEX-NMCS2 vector, donated by Dr. Craig Neville at Massachusetts General Hospital (Rosenthal et al., 1990; Neville et al., 1996). pMEX-NNCS2 contains rat myosin light chain 1 (MLC1) regulatory sequences (1.5 Kb), SV40 splice/poly adenylation fragment (0.8 kb), and MLC enhancer (0.9 kb). The ribozyme construct was cloned into the Sal I/Hind III site of the vector pMEX-NNCS2. The 3.6 kb construct (MLC-Rib) was obtained by Not I digestion of the vector. The pro domain transgene was inserted into the EcoRI /Hind III site of pMEX-NMCS2 vector. The recombinant vector pMEX-NMCS2 was then digested with Not I restriction enzyme, resulting in the 4.2 kb construct (MLC-Pro; Fig. 1). Both transgene constructs includes MLC1 promoter, SV40 Poly adenylation sequence, and MLC enhancer in addition to the transgene sequence (ribozyme multimer or pro domain DNA sequence).

Microinjection and detection. Transgenic mice were generated by standard pronuclear microinjection techniques using B6SJL F1 females as zygote donors and CB6 females as embryo recipients. The experimental protocols for this research were approved by the Beltsville Area Institutional Animal Care and Use Committee. Presence and copy number of transgenes in the founder mice were determined by Southern blotting, where a 0.3 kb DNA sequence of the ribozyme multimer was used as a probe for the MLC-rib construct, and an 1.1 kb DNA sequence containing the 0.8 kb myostatin pro domain DNA fragment and a partial SV40 poly adenylation sequence was used as a probe for the MLC-pro construct.

### **Detection of Transgene Expression**

Total RNA was isolated from Gastrocnemius muscle (50-100~mg) with TRIzol reagent (Gibco-BRL, Gaitherburg, MD). The RNA level of the MLC-rib transgene was detected by Northern blotting. The membrane was stripped and re-probed with human  $\beta$ -actin cDNA to normalize sample loading. The signal intensity was determined by a phosphorimager (STORM 860, Molecular Dynamics, Sunnyvale, CA). The ratio of transgene mRNA to  $\beta$ -actin mRNA was used to compare levels of transgene expression between different lines.

### **Growth and Carcass Evaluations**

Nontransgenic sex-matched littermates were used as controls for comparison with transgenic mice. Both transgenic mice and their sex-matched controls were housed in the same cage. Mice were weaned at 30 days of age, and weighed at 5-days intervals from birth to weaning, then weekly from 4–9 weeks, and monthly thereafter.

Because this study was considered a model for a transgenic pig project, a similar carcass dissection method as one used on pig carcasses was adapted to evaluate the total muscle mass. Mice were killed at 6, 9, and 18 weeks of age. They were skinned and trimmed by removing the head at the 7th cervical vertebra (C7), the paws from tarsals and carpals, and the tail from the 3rd sacral vertebra (S3). After obtaining carcass weight, hind limbs were removed from the last lumbar vertebrae, fore limbs were separately removed at the edge of scapula bone, and the remaining carcass was referred to as the trunk. The hind limbs, fore limbs, and trunk were weighed separately. Internal organs including heart, lung, kidney, spleen, and liver were weighed, as was the epididymal fat in males.

### Determination of Muscle Fiber Size and Number

Four transgenic and four nontransgenic littermate male mice were used to study the effects of transgene expression on myofiber size and number. The left hind limbs were removed within 10 min of killing the animals at 9 weeks, and the paws were removed. Samples were quickly dipped in liquid nitrogen for 5 sec and then stored at  $-70^{\circ}$ C. Frozen muscle samples were equilibrated at  $-25^{\circ}$ C, and 0.5 cm of the limbs from the distal end was removed. Muscle samples were perpendicularly mounted on a cryostat chuck by the knee joint with tissue freezing medium (Triangle Biomedical Science, Durham, NC). Sections 8 µm thick were cut using a Cryostat 2800 Frigocut-E (Reichert-Jung, Cambridge Instrument, NY), and mounted on glass slides. The sections were treated with a combination of succinic dehydrogenase and acid myofibrillar ATPase staining procedure (Solomon and Dunn, 1988).

Stained sections were observed at  $200\times$  with a Zeiss microscope in transmitted light mode (Carl Zeiss, NY). Muscle fibers were classified according to Peter et al. (1972) into slow-twitch oxidative (SO, Type I or red

fiber), fast-twitch oxidative glycolytic (FOG, Type IIA, or intermediate) and fast-twitch glycolytic (FG, Type IIB, or white). The SO fibers showed the darkest, FG the lightest, and FOG the intermediate staining intensity. Four images at different locations within one section were captured for gastrocnemius/plantaris (GAS/PL) and tibialis cranial, respectively, with a Kontron 300 image analysis system (Kontron Elektronik Corp., Newport Beach, CA). Two images were captured for soleus muscle. Fiber cross sectional area (μm<sup>2</sup>) was calculated by dividing the area of multiple adjacent fibers by the number of fibers for each type within each image. On the basis that gastrocnemius, tibialis cranial, and soleus muscle mainly consists of FG, FOG, and SO fibers, respectively, the fiber cross sectional area of FG, FOG, and SO was correspondingly obtained from its respective muscle. The total fiber number of the cross section from GAS/PL muscle was estimated as based on the cross section area and the average cell number of the four captured imagines.

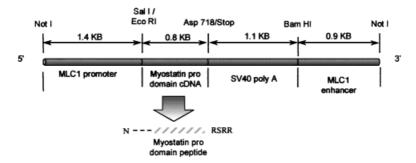
### **Statistical Analysis**

The RNA levels of the MLC-pro transgene were expressed as a ratio of transgene mRNA to β-actin mRNA by band density. Ratios were transformed before being subjected to GLM analysis (SAS Inst., Inc., Cary, NC), where transgene-expressing level was the main effect of treatment. Pearson's correlation comparing expression levels and weights were calculated. In the studies of growth performance, body weights at all time points between transgenic and nontransgenic controls were analyzed by two-tailed Student's t-tests. Preliminary statistical analysis of live and carcass weights indicated that the phenotypic response to the presence of the MLC-pro transgene differed between males and females (P < 0.038). Therefore males and females were analyzed separately. In the GLM models Transgene and expressing levels were treated as the main effects. In the study of effects of transgene on fiber size and number, four images from one animal were treated as four replicates with transgene as the treatment effect in one-way ANOVA. Least square means and standard error of the means are presented in this report.

### RESULTS

### Transgenic Mice With the MLC-Rib Construct

The multimer ribozyme construct, placed under transcriptional control of the skeletal muscle-specific promoter and enhancer elements from the rat MLC1 gene was injected into fertilized mouse eggs. Among the 124 pups resulting from microinjected eggs, 15 transgenic founder mice were identified by Southern blotting of mouse tail DNA. Ribozyme RNA expression was detected in six independent lines by Northern blotting. Transgenic mice from all lines appeared normal and healthy. The live weight of transgenic mice from expression lines at 9 weeks was not significantly different from their sex-matched littermate controls



**Fig. 1.** MLC-pro transgene construct. The MLC-pro construct consists of myosin light chain (MLC) promoter, partial myostatin cDNA from the 5′ untranslated region to the nucleotides encoding the prodomain cleavage site, RSRR, a SV40 PolyA tail and MLC1 enhancer.

(male  $25.22\pm0.49$  vs.  $25.40\pm0.70$  g, P=0.83; female  $21.58\pm0.66$  vs.  $22.32\pm0.14$  g, P=0.27). Carcass analysis was not performed because of the lack of growth differences attributed to the transgene.

### Transgene Expression in the MLC-pro Mice

Among 156 pups resulting from MLC-pro microinjected eggs, 25 transgenic founder mice were identified by Southern blotting of mouse tail DNA. Offspring from nine founders were shown to express the transgene in their skeletal muscles by Northern blot analysis. Levels of transgene expression were normalized by dividing by the level of β-actin mRNA in skeletal muscle. Three independent lines with HIGH (Ratio of pro domain to βactin mRNA: 0.052), MEDIUM (0.031), and LOW (0.0052) levels of transgene expression were established to study in detail. A Northern blot of the gastrocnemius muscle RNA of the offspring from these three lines is shown in Fig. 2. The levels of transgene expression differed among these three lines (P < 0.001). The ratio of transgene to β-actin mRNA in the HIGH and MEDIUM expressing lines is about ten and six times higher than that of the LOW expressing line, respectively. HIGH, MEDIUM, and LOW expressing lines had 3, 3, and 15 copies of the transgene per genome, respectively, as determined by Southern blotting analysis. Level of transgene expression was not correlated with transgene copy number. By Northern blotting, we did not find any detectable signals of transgene RNA in other tissues including adipose fat, brain, heart, intestine, kidney, liver, lung, spleen, testis, and uterus.

### Growth Performance of the MLC-pro Transgenic Mice

Growth curves of transgenic and littermate control mice from the HIGH expressing line are shown in Fig. 3. All mice were weaned on the 30th day of age. At 25 days of age, a difference in weight between males and females could be detected (14.13  $\pm$  0.24 vs. 12.49  $\pm$  0.46 g, P = 0.0115). This growth difference between the sexes resulted in a significant Sex by Transgene interaction (P = 0.0126), and therefore, the sexes were analyzed

separately. A difference in weight caused by the transgene was not detected until 30 days of age. By day 30, the weight of transgenic males was greater than that of their nontransgenic littermates (19.82  $\pm$  0.74 vs.  $17.53 \pm 0.77$  g, P = 0.0241). However, transgenic females did not outperform their littermate controls until day 37 (live weight:  $20.4 \pm 1.04$  vs.  $18.24 \pm 0.41$  g, P = 0.0163). Transgenic males were 20-46% heavier than littermate controls from day 30 to 4 months (P < 0.05). Whereas, transgenic female mice were 10-20% heavier than littermate controls from day 37 to 4 months (P < 0.05). Collectively this data demonstrate that gross weights of transgenic mice are not different from their littermate controls before weaning, but phenotypic differences between transgenic and control mice were only apparent at/or beyond one month of age.

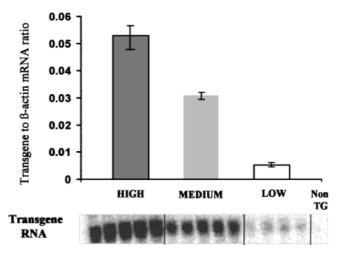
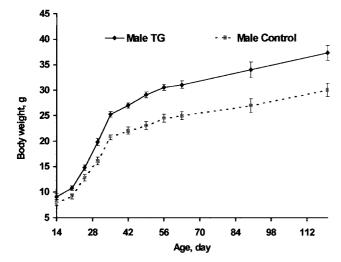
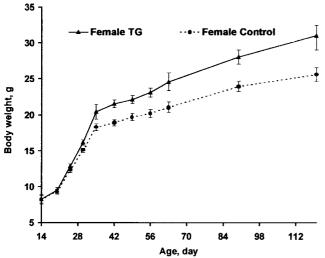


Fig. 2. MLC-pro transgene expression in the gastrocnemius muscle of three transgenic lines. Total RNA was isolated from gastrocnemius muscle from male offspring of HIGH (lanes 1–5), MEDUM (lanes 6–10), and LOW (lanes 11–14) expressing lines, and a nontransgenic littermate (lane 15) was used as a control. Northern blotting was carried out using a myostatin pro domain cDNA fragment as a probe. The membrane was stripped and re-probed with human β-actin to correct for the sample loading (bands not shown). The band density was quantified, and levels of transgene expression were expressed as ratio of transgene mRNA to β-actin mRNA. Levels of transgene expression differed among lines (P < 0.01).





**Fig. 3.** Growth curves of transgenic and littermate controls from HIGH expressing line. Mice were weighed at the indicated ages. The growth curves of male mice were based on 6 transgenic and 6 littermate control mice, and female mice growth curves were derived from 6 transgenic and 10 littermate control mice.

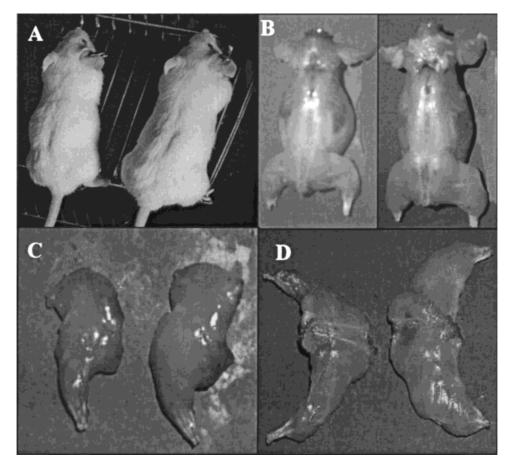
### Carcass Weight of the MLC-pro Transgenic Mice

Transgenic mice, especially from the HIGH expressing line, appeared larger, and exhibited pronounced muscling in the hips and shoulders (Fig. 4A,B). Carcasses were dissected into three parts; hind limbs, fore limbs, and trunk for muscular phenotype evaluation at 6, 9, and 18 weeks of age. Preliminary statistical analysis of carcass weights indicated that the phenotypic response to the presence of the MLC-Pro transgene differed between males and females (P < 0.038 for interaction of Transgene and Sex). Therefore, males and females were analyzed separately. The live and carcass weights at 9 weeks is summarized in Table 1, and the weight data from 6 and 18 weeks were graphed in Figs. 5 and 6. The presence of the transgene resulted in an increase in weights of whole carcass, trunk, hind limbs, and fore limbs at 9 weeks in the HIGH and MEDIUM expressing lines. However, carcass weight measurements in LOW line mice did not differ from controls (P>0.05), Transgene by Line interaction, Table 1). A similar Transgene by Line interaction was detected for males at 6 and 18 weeks of age (Figs. 5 and 6), and was mostly attributable to enhanced phenotype observed in HIGH and MEDIUM lines compared to the LOW expressing line.

Increases in carcass weights of transgenic males were substantial. The whole carcass weights of transgenic males from the HIGH line were 39, 45, and 48% greater than those of their littermate nontransgenic controls at 6, 9, and 18 weeks, respectively (P < 0.001). The increases in fore limb weights were even more impressive (45, 62, and 60% increase at 6, 9, and 18 weeks, respectively).

In males, there tended to be a direct linear relationship between levels of expression of the transgene and the degree of exhibited phenotype. Percentage increases in carcass weight of transgenic males over their littermate controls at 9 weeks of age were 45, 30, and 16 for HIGH, MEDIUM, and LOW expressing lines, respectively (P < 0.0001, Table 1). Hind and fore limbs and trunk weights showed similar increase in transgenic mice over controls in these three lines. To quantify the relationship between the transgene expression and muscular phenotype, transgene expression in individual animals was normalized against β-actin expression and compared to mouse weights. The normalized level of expression of the transgene was not correlated with live body weight (r = 0.565, P = 0.0556), but highly correlated to carcass weights (r=0.722,P = 0.008), which accounts for approximately half of the variation observed.

In females, the relationship between levels of transgene expression and muscular phenotype was not as dramatic as in males. As with the males, the LOW expressing line's phenotype was the least pronounced. However, enhancement in whole body, carcass, hind limbs and fore limbs, and trunk in females was more similar between HIGH and MEDIUM lines than was seen for males. In females at 6 and 9 weeks of age, there was an interaction between Transgene and expressing levels for all parameters measured (P < 0.05). At 6 weeks of age, females from the LOW line did not differ from their nontransgenic littermates. At 9 weeks of age, the difference in muscle mass between female transgenic and control mice was moderate (26% increase in trunk weight) to substantial (56% increase in fore limb weights) for the HIGH line (P < 0.001, Table 1). The increases were less pronounced in the MEDIUM line ranging from a 4% increase in live weight to a 22% increase in fore limbs and trunk (P < 0.01). At 18 weeks of age, response of females to the transgene was more consistent among lines (Figs. 5 and 6). The interaction of Transgene by expression levels in females for all the parameters measured were not significant at 18 weeks of age (P > 0.1), demonstrating that effects of the transgene in 18-week old females were significant in all three levels of transgene expression (P < 0.0001).



**Fig. 4.** Phenotype of transgenic and littermate control mice from HIGH expressing line. Transgenic mice and littermate control are shown on the left and right, respectively, for the live animals (A), whole carcasses (B), fore limbs (C), and hind limbs (D).

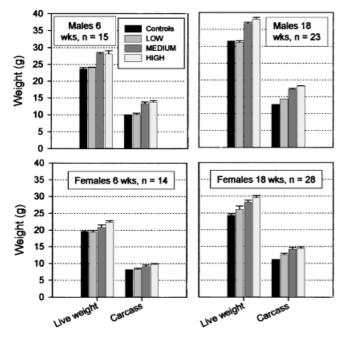
### Effects of the MLC-Pro Transgene on Muscle Fiber

To determine whether increased muscle mass in transgenic mice was related to muscle fiber hypertrophy, hyperplasia, or both, we further studied muscle fiber cross-sectional area and fiber number using male mice from the HIGH expressing line. Three types of muscle fibers, namely FG, FOG, and SO, were clearly identified in each section. The cross-sectional area of FG fiber of the gastrocnemius muscle, which is also referred to as Type IIB fiber, was larger in transgenic mice than in littermate controls (P < 0.001, Fig. 7). Transgenic mice also showed larger cross-sectional

TABLE 1. Live and Carcass Weight of Transgenic Mice and Littermate Control at 9 Weeks of Age (Mean  $\pm$  SEM)

Expression level (lines)							P values		
Sex	$\mathrm{Obs}^1$	Items	HIGH	MEDIUM	LOW	$\mathrm{Controls}^2$	Trans- gene	Level	$T{ imes}L$
Males Females	28 32	Live weight Carcass Trunk Hind limbs Fore limbs Live weight Carcass Trunk Hind limbs For limbs	$\begin{array}{c} 32.11\pm1.00\\ 16.05\pm0.67\\ 6.94\pm0.29\\ 6.59\pm0.22\\ 2.52\pm0.10\\ 22.78\pm0.75\\ 9.99\pm0.38\\ 4.2\pm0.31\\ 4.28\pm0.14\\ 1.48\pm0.06 \end{array}$	$\begin{array}{c} 29.53 \pm 0.51 \\ 14.19 \pm 0.24 \\ 6.17 \pm 0.20 \\ 5.82 \pm 0.09 \\ 2.19 \pm 0.05 \\ 22.74 \pm 0.31 \\ 9.98 \pm 0.12 \\ 4.31 \pm 0.11 \\ 4.18 \pm 0.09 \\ 1.49 \pm 0.04 \end{array}$	$\begin{array}{c} 28.63 \pm 0.52 \\ 12.65 \pm 0.34 \\ 5.36 \pm 0.18 \\ 5.45 \pm 0.17 \\ 1.86 \pm 0.08 \\ 21.46 \pm 0.46 \\ 9.14 \pm 0.26 \\ 3.98 \pm 0.12 \\ 3.87 \pm 0.12 \\ 1.33 \pm 0.06 \end{array}$	$\begin{array}{c} 26.00 \pm 0.56 \\ 11.16 \pm 0.28 \\ 4.88 \pm 0.12 \\ 4.62 \pm 0.17 \\ 1.59 \pm 0.05 \\ 20.85 \pm 0.30 \\ 8.19 \pm 0.19 \\ 3.69 \pm 0.09 \\ 3.33 \pm 0.09 \\ 1.17 \pm 0.025 \end{array}$	$\begin{array}{c} <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ <0.0001\\ \end{array}$	0.5193 0.0169 0.0459 0.1064 0.0170 0.3724 0.0226 0.6932 0.0514 0.3296	0.0019 0.0011 0.0021 0.0022 0.0002 0.0006 0.0002 0.0296 0.0001 0.0351

 $<sup>^{1}</sup>$ A minimum of three transgenic animals along with at least one sex matched, littermate control were weighed for each line.  $^{2}$ Control weights did not differ between lines for any parameter measured (P > 0.078) and therefore control weights were pooled for clarity of presentation.

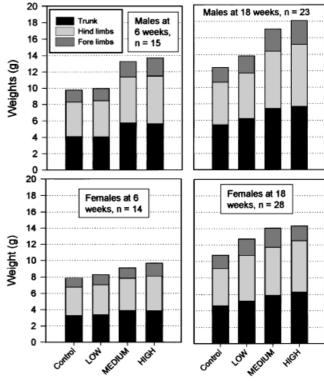


**Fig. 5.** Live and carcass weight of transgenic and sex-matched littermate control mice from the three expressing lines at 6 and 18 weeks. Weights for control mice from different lines did not differ (P > 0.05) and therefore control weights were pooled for clarity of presentation. Bars represent the least square mean weights and SEM for the controls, and LOW, MEDIUM, and HIGH transgenic lines.

area of FOG muscle fibers from tibialis cranial than their littermate controls (P < 0.01). However, the cross-sectional area of SO muscle fibers from soleus were not different between transgenic and control mice (P > 0.05). The total number of fibers in gastrocnemius muscle was not different between transgenic mice (4986.7  $\pm$  678.3) and littermate controls (5284.6  $\pm$  493.7, P > 0.05). Collectively, the increased muscle mass by this transgene most likely resulted from increased fiber cross-sectional size.

## Effects of the MLC-Pro Transgene on Epididymal Fat, Heart, and Other Organs

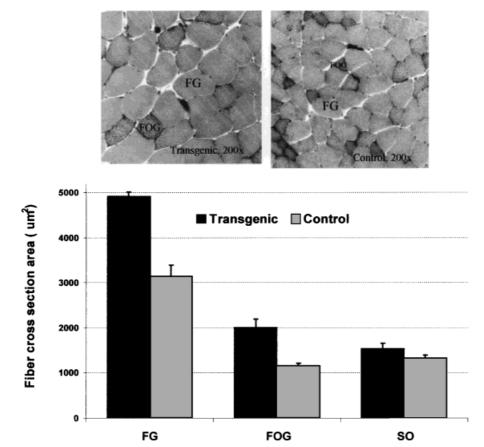
It is well documented that double muscled cattle have lower fat content in their carcass (Arthur, 1992). Mouse body fat is highly correlated with epididymal fat pad (Eisen and Prasetyo, 1988). To determine if the transgene influenced body fat deposition, epididymal fat pad weights were taken at 6, 9, and 18 weeks of age for transgenic and littermate control mice. At 6 weeks of age, the weight of epididymal fat was significantly lower (P < 0.05) for mice from the HIGH line  $(0.216 \pm 0.011 \text{ g})$  when compared with weights from the MEDIUM line  $(0.245 \pm 0.012 \text{ g})$ , and LOW line  $(0.253 \pm 0.010 \text{ g})$ . Epididymal fat weights did not differ between the MEDIUM and LOW lines (P = 0.5771). At 9 weeks of age, epididymal fat weights were lower in the transgenic mice of HIGH line than that in controls  $(0.162 \pm 0.014 \text{ vs. } 0.315 \pm 0.020 \text{ g}, P < 0.0001)$ , and were inversely related to the levels of transgene expression



**Fig. 6.** Weights of carcass components (trunk, hind, and fore limbs) from transgenic and sex-matched littermate control mice at 6 and 18 weeks. Weights for control mice from different lines did not differ (P>0.05), and therefore control weights were pooled for clarity of presentation. The stacked bars represent the least square mean weights. The height of the bars (sum of the components) differs from the carcass values presented in Fig. 5 only by experimental error.

(HIGH,  $0.162\pm0.014$  g; MEDIUM,  $0.223\pm0.015$  g; LOW,  $0.307\pm0.017$  g, P<0.0001). By 18 weeks of age, levels of transgene expression did not influence epididymal fat weight (P=0.0614), but the epididymal fat weights were reduced in transgenic mice in comparison with their littermate controls in all three lines  $(0.405\pm0.024~{\rm vs.}~0.506\pm0.029~{\rm g}, P=0.0052)$ .

In all three lines, heart weight was not different between transgenic and nontransgenic sex-matched littermate controls at 6, 9, and 18 weeks of age. For example, at 9 weeks age, the heart weights of the HIGH line between transgenic and littermate controls were not different (male:  $0.18 \pm 0.015$  vs.  $0.17 \pm 0.015$  g, P = 0.38; female:  $0.14 \pm 0.008$  vs.  $0.12 \pm 0.015$ P = 0.15). In most mammals, heart weight is proportional to body weight (r=0.99 in 104 mammalian)species; Prothero, 1979). There is a tendency for double muscled cattle to have smaller internal organs (Arthur, 1992). In all three tested MLC-pro transgene expressing lines, heart as a percentage of body weight was not different between transgenic and nontransgenic sexmatched littermate controls at 6 and 9 weeks of age. However, at 18 weeks of age, heart as a percentage of body weight was less than controls by  $\sim 20\%$  for males  $(0.62 \pm 0.02 \text{ vs. } 0.75 \pm 0.03\%, P = 0.0025)$  and 10% for females  $(0.68 \pm 0.033)$  vs.  $0.77 \pm 0.02\%$ ,



**Fig. 7.** Comparison of fiber cross-sectional area between transgenic and littermate control mice. Muscle samples were taken from the left hind limb of 4 transgenic and 4 littermate controls from the HIGH expressing line. A transverse cryostat section of the GAS/PL was histochemically stained. The top panels are the cross sections of GAS/

PL muscles from a transgenic mouse and its littermate control. Means and SEM of fiber cross-sectional area of FG, FOG, and SO fibers were obtained from GAS/PL, tibialis cranial, and soleus, respectively. FG and FOG fiber sizes differed between transgenic mice and littermate controls (P < 0.01).

P = 0.033). Furthermore, at 18 weeks of age the heart weight of males and females from three lines with different expressing levels responded differently (P = 0.0399 for expressing level  $\times$  sex interaction). Heart as a percentage of body weight for females was not different between transgenic mice and controls in the MEDIUM and LOW expressing lines though heart was a smaller percentage of body mass for the HIGH expressing line. The transgenic males of the HIGH expressing line exhibited smaller hearts relative to body mass than controls  $(0.63 \pm 0.02 \text{ vs. } 0.77 \pm 0.04\%,$ P < 0.05). In addition, transgenic male mice from the LOW expressing level also had relatively smaller hearts  $(0.64 \pm 0.003 \text{ vs. } 0.77 \pm 0.06\% \ P < 0.05)$  at 18 weeks of age. Thus, the transgene increased live body and carcass weights, but did not result in larger hearts in relation to their larger body size based on comparison to the littermate control mice.

We also collected weight data for spleen, liver, lung, and kidney. Solid organ weights from transgenic mice, and an equal number of sex-matched littermate controls were statistically compared within each line at 18 weeks of age. The presence of the transgene did not significantly influence the weight of liver

 $(1.71\pm0.06 \text{ vs. } 1.75\pm0.05 \text{ g, } P=0.53)$ , lung  $(0.34\pm0.014 \text{ vs. } 0.35\pm0.017 \text{ g, } P=0.52)$ , or spleen  $(0.10\pm0.003 \text{ vs. } 0.114\pm0.05, P=0.09)$ . However, when organ weights were adjusted for live body weight, the livers  $(5.49\pm0.22 \text{ vs. } 6.4\pm0.24\%, P=0.0043)$  and lungs  $(1.09\pm0.04 \text{ vs. } 1.29\pm0.06\%, P=0.002)$  from transgenic animals were significantly smaller than those in the control animals. The transgene had no detectable effect on kidney weight (P=0.77), though there was a difference in kidney weight among mice from different expressing levels and between sexes (P<0.0001 and P<0.001). Analysis of kidney weight on a per body weight basis did not reveal an effect of the transgene (data not shown).

### Reproduction of the MLC-Pro Transgenic Mice

The MLC-pro transgenic mice from all lines appeared normal, healthy, and they reproduced well without fertility or litter-delivery problems. The litter size of transgenic mice from MLC-pro lines were similar to the average litter size resulting from contemporary matings of nontransgenic mice in our colony (nontransgenics:  $8.27 \pm 0.28$  pups/L, n=22 litters vs.  $8.69 \pm 0.20$ 

pups/L, n=42 litters, P>0.45). Litter size was not different among the studied three expressing lines (P>0.9).

### **DISCUSSION**

### **Transgenics for Increasing Muscle Growth**

The potential application of transgenic technology for livestock production was first demonstrated in transgenic mice carrying a human growth hormone (GH) transgene (Palmiter et al., 1982). Since then, the hormone cascade from growth hormone-releasing factor (GRF) and GH to insulin-like growth factor I (IGF-I), has been the target of transgenic projects designed to increase livestock growth and carcass quality (Pursel et al., 1989). In transgenic mice with GH from rat, human, and bovine, growth rates (5-11 weeks) were increased by four-folds. Transgenic pigs generated with a bGH transgene construct showed a significant increase in feed efficiency and a reduction in backfat thickness (Pursel et al., 1989, 1997). However, various animal health problems such as hepatomegaly, glomerular sclerosis, osteochondritis, and infertility in females were observed in those pigs. Transfers of GRF and c-ski transgenes have also been tested in livestock animals (Pursel et al., 1990; Bowen et al., 1994), but neither construct resulted in useful phenotypes. Recently, transgenic pigs carrying an IGF-I transgene have been generated. Lean tissue growth in the transgenic pigs was significantly improved with 10–20% larger loin eye area and lower body fat percentage in comparison with nontransgenic littermate controls (Pursel et al., 1999; Bee et al., 2001).

Animal muscle development is not only regulated by systemic circulating hormones, but also by tissuespecific effectors influencing muscle fiber development such as myostatin. Mouse myostatin cDNA encodes a protein of 376 amino acids that is cleaved to produce an active protein of 109 amino acids, which negatively regulates both myoblast proliferation and fiber development (McPherron et al., 1997; Thomas et al., 2000). Directly targeting this kind of local effector with transgenic technology might offer more flexibility in controlling muscle development without adverse effects on other tissues. In consideration of potential applications of down-regulation of myostatin to improve muscle development in swine, we tested the feasibility of ribozyme and myostatin pro domain cDNA transgene constructs to depress myostatin activity in transgenic mice.

### Transgenics With the Ribozyme Construct

Transgenic mice based on the ribozyme construct did not appear different from nontransgenic littermate controls. Their body weights at 9 weeks of age were very similar in both sexes, suggesting that the ribozyme construct was unable to interfere with myostatin mRNA. It is not well understood that how endogenous myostatin mRNA expression is regulated in the skeletal muscle.

In this study, ribozyme mRNA was detected in the skeletal muscle of MLC-rib transgenic mice without apparent influence on muscle development. However, the efficiency of ribozyme activity and the level of myostatin RNA in the skeletal muscle of transgenic mice were not studied. It is possible that the RNA transcribed from the construct was produced at an inadequate amount so that it did not bind effectively with its target myostatin mRNA. This experiment, as with numerous other ribozyme-based transgenic projects, failed for unknown reasons.

### **Transgenic Mice With Depressed Myostatin**

The observed phenotype in transgenic MLC-pro mice demonstrated that transgene constructs with a myostatin cDNA pro domain fragment worked effectively in promoting muscle growth and mass without causing any obvious health problems in mice. In the HIGH expressing line, body weight increased by  $\sim 20\%$  and carcass weight increased by ~40% at 9 weeks of age in both sexes. Live weights at 4 months from the HIGH and MEDIUM expressing lines described here were intermediate to heterozygous and homozygous myostatin-knockout mice in both males and females (McPherron et al., 1997). Our carcass data are not directly comparable with the knock-out mice data due to different methods used for carcass dissection. Weights of individual muscles of homozygous knockout mice were 100-200% higher than those of wild-type mice. Carcass weights of homozygous knockout mice, however, were only 50% higher than those of wild-type at 4 months mice, which is similar to the reported percentage increase in carcass weights of transgenic mice over their littermate controls in the HIGH and MEDIUM expressing lines. The muscular phenotype of MLC-pro transgenic mice is also similar to mice expressing a mutant form of myostatin (Zhu et al., 2000). In that study, levels of active myostatin protein in skeletal muscle were reduced, resulting in increased muscle mass by 20-35% in gastrocnemius, triceps, and quadricep muscles in comparison to littermate controls. Therefore, dramatic muscular phenotypes can be generated with transgenes directed against myostatin function either by over-expression of its pro domain, preventing pro domain cleavage, or by knocking out myostatin expression.

### Levels of the MLC-Pro Transgene Expression and Muscular Phenotype

Interestingly, levels of the MLC-pro transgene expression in this report corresponded well with growth and muscular phenotypes in the three tested expressing lines. The HIGH expressing line had the most exaggerated phenotype. The mice from the MEDIUM expressing level showed less dramatic growth and muscle accretion, and mice from the LOW expressing line deviated least from controls. There seemed an obvious relationship between levels of transgene expression and magnitude of the phenotype observed.

In transgenic mice expressing myostatin mutated at its cleavage site, levels of both myostatin RNA and protein were reduced (Zhu et al., 2000). We speculate that the amount of transgene mRNA we detected is likely correlated with the amount of myostatin pro domain protein in our transgenic mice. Like the live weight, the muscular and carcass phenotypes of the HIGH expressing line were also intermediate between heterozygous and homozygous myostatin knock-out mice, where either 50% or no functional myostatin was present in the muscle. Therefore, functional myostatin is probably still present in the MLC-pro transgenic mice, and the muscular phenotype results from partially blocking myostatin biological activity.

### Myofiber Characteristics of Myostatin Dominant-Negative and Knock-Out Mice

In the myostatin knock-out mice, increased muscle mass results from muscle hyperplasia as well as hypertrophy (McPherron et al., 1997). The enlarged muscles in Belgian Blue cattle are not the result of muscle fiber hypertrophy, but primarily that of muscle fiber hyperplasia, as demonstrated in a recent study (Wegner et al., 2000). That study showed that fiber cross-sectional area in the semitendinosus muscle was not significantly different between Belgian Blue and nondouble-muscled cattle from birth to 2 years of age. However, muscle fiber number is much higher in Belgian Blue at birth than in nondouble-muscled cattle, and the number of fibers does not change during postnatal development. A myofiber hypertrophy without hyperplasia was found to be solely responsible for the increased muscle mass in the transgenic mice carrying a cleavage site-mutant myostatin (Zhu et al., 2000). In our transgenic mice with over-expressed myostatin pro domain, we found that myofiber number in the gastrocnemius/plantaris did not increase, but an increase in fast-twitch fiber size was observed

Fiber number is known to be determined during embryonic development and does not change postnatally (Luff and Goldspink, 1970; Ashmore et al., 1974; Wegner et al., 2000). Recent studies have shown that a high level of myostatin mRNA is present in fetal muscle (Bass et al., 1999), and myostatin inhibits myoblast cell proliferation in C2C12 cell culture (Thomas et al., 2000). These data suggest that myostatin possibly plays a critical role in determination of the number of myoblast cells that would differentiate into muscle fibers instead of regulation of myoblast differentiation process during fetal development. We did not find a detectable signal for transgene RNA in 14-day old fetus (data not shown). Based on transcriptional activation of MLC1, locus in skeletal muscle occurs at ~4 days before birth (Periasamy et al., 1984), it is possible that the expression of the pro domain of myostatin by the MLC1 promoter is not strong enough during embryonic development to block myostatin activity. The increased muscle mass in the Belgian Blue cattle and myostatin knock-out mice corresponds well with myofiber hyperplasia, which is apparently attributable to myostatin

mutation, and consequently absent of myostatin during early fetal development. The fact that blocking myostatin activity by pro domain transgene was not able to produce hyperplasia, may result from low MLC1 promoter activity due to the time of MLC gene expression at later stage of fetal development.

Reporter genes directed by either MLC1f or MLC3f promoters exhibit skeletal muscle-specific expression in transgenic mice, and are preferentially expressed in the fast twitch fibers (Neville et al., 1996). Our data is consistent with those observations. Fiber type FG corresponds to the fast-twitch, SO to the slow-twitch fibers, and FOG is the metabolic intermediate fibers of FG and SO fibers. It is therefore likely that the increased fiber size of FG and FOG, and lack of affect on SO fiber size is a function of the differential expression pattern of MLC1 promoter.

In conclusion, transgenic mice carrying the myostatin pro domain construct exhibited dramatic muscle development with increased growth performance and carcass weight. The magnitude of the observed phenotype was positively correlated with measured levels of transgene expression. Transgenic mice did not display the muscular phenotype until one month of age, and they appeared normal, healthy, and without reproduction problems. Therefore, this mouse model suggests that a transgenic pig project based on myostatin pro domain construct would be appropriate for potential improvement of pig muscle development.

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